

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

## IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

PCT

WORLD INTELLECTUAL PROPERTY  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER

WO 9605293A1

(51) International Patent Classification <sup>6</sup> :	A1	(11) International Publication Number: WO 96/05293 (43) International Publication Date: 22 February 1996 (22.02.96)		
(21) International Application Number:	PCT/EP95/03114			
(22) International Filing Date:	4 August 1995 (04.08.95)			
(30) Priority Data:	1545/94 8 August 1994 (08.08.94)	AT		
(71) Applicant (for all designated States except US):	UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION [AT/AT]; Vienna International Centre, A-1140 Vienna (AT).	(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).		
(72) Inventors; and		Published		
(75) Inventors/Applicants (for US only):	BARALLE, Francesco, Ernesto [AR/IT]; Area Science Park, Padriciano, 99, I-34012 Trieste (IT). SCODELLER, Eduardo [AR/IT]; Area Science Park, Padriciano, 99, I-34012 Trieste (IT). TISMINETZKY, Sergio [AR/IT]; Area Science Park, Padriciano, 99, I-34012 Trieste (IT).	With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.		
(74) Agents:	ITZE, Peter et al.; Amerlingstrasse 8, A-1061 Vienna (AT).			
(54) Title:	MOLECULAR PRESENTING SYSTEM			
(57) Abstract	The invention refers to a molecular presentation system in which viral proteins are foreseen as carriers for heterologous amino acid sequences. Hereto, the viral protein is derived from small insect viruses, primarily from Flock House Virus (FHV), with a known 3-dimensional structure and amino acid sequence, whereby heterologous amino acid sequences, for example epitopes, are inserted in the outwards directed loops of the viral capsid protein. Moreover, the expression of the FHV capsid protein in insect cells can produce mature virus like particles (VLP) through a recombinant baculovirus.			
FHV Capsomer virus-like particles produced in Baculovirus.				
<pre>graph TD     BACULOVIRUSDNA[BACULOVIRUSDNA] --&gt; POLYHEDRIN[POLYHEDRIN PROMOTER]     BACULOVIRUSDNA --&gt; FHVINSERT[FHV INSERT]     POLYHEDRIN --&gt; TRANSCRIPTION[TRANSCRIPTION]     FHVINSERT --&gt; TRANSCRIPTION     TRANSCRIPTION --&gt; FHVmRNAI[FHV mRNA I]     TRANSCRIPTION --&gt; FHVmRNAA[FHV mRNA II]     FHVmRNAI --&gt; TRANSLATION[TRANSLATION]     FHVmRNAA --&gt; TRANSLATION     TRANSLATION --&gt; COATPROTEIN[COAT PRECURSOR PROTEIN]     COATPROTEIN --&gt; VLP[V.L.P.]</pre>				

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Larvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

Molecular presenting system

The invention concerns a molecular presentation system in which viral proteins are 5 being used as carriers for heterologous amino acid sequences.

The possibility to identify and synthesize amino acid sequences from viral proteins, which are able to generate a protective immune response in animals, has stimulated the development of synthetic vaccines. Although it has already been shown that synthetic peptides in some cases can induce a good immune response, it has turned 10 out that in general they were weak immunogens unless coupled to strongly immunogenic carrier molecules. They were frequently unable to induce protective immunity in vaccinated animals. Attempts to increase the immunogenicity of these antigens for use as vaccine have lead to the development of a series of antigen presentation systems. Many of these are designed to present the antigen as a polyvalent, particulate structure. The 15 development of particulate vector systems for immunogenic epitopes provides a powerful approach for the presentation of antigens. Various systems were used to present foreign epitopes: the core antigen of Hepatitis B virus (HBV) (HBcAg) [1] and the surface antigen of Hepatitis B virus (HBsAg) [2], the capsid protein from Polio virus [3], the yeast Ty protein [4], the particles obtained after insertion of HIV 1-gag in Baculovirus 20 [5], rotavirus VP-6 protein [6], core particles of the Bluetongue virus (BTV) [7], and filamentous as well as icosahedral bacteriophages [8,9].

It has been demonstrated that the immunogenicity of a peptide depends on its sequence as well as on the way it is presented to the immune system. By using a human rhino virus capsid sequence as a heterologous peptide and the particles of HBcAg as a 25 carrier, it was shown that the internal location of the foreign sequence increases the immunogenicity of the epitope by 10 to 50 fold when compared to the amino terminus location [10]. Also the antigenicity (measured as reactivity to a monoclonal antibody (mAb)) was greatly enhanced by placing the foreign peptide in that position in the carrier. Furthermore, both constructs presented the epitopes considerably more efficiently to the 30 mAbs than the free peptides. This was also the case when specific HIV-1 epitopes (the V3 loop) were introduced into different domains of the HBcAg [11]. Since the properties

of a given epitope can be influenced by its conformation it was of great interest to have a carrier system with multiple entry sites conferring many possible conformations. This would increase the possibility of finding a conformation closer to the native one for a given sequence. In spite of the fact that, as mentioned above, various particulate systems 5 have been developed for the presentation of epitopes, they were all based on the foreign epitope being inserted mainly in one position. This was partly due to lack of knowledge about the 3-D structure of the carrier particle.

#### Summary of the invention.

10

With reference to the above, a new presentation system has been developed, characterized by the fact that the carrier protein is derived from small insect viruses, Flock House virus (FHV), with a known 3-D structure and amino acid sequence. Heterologous amino acid sequences, for example epitopes, are inserted into the outwards 15 directed loops of the viral capsid protein. This carrier presents multiple possibilities for a conformationally suitable location of epitopes. Above all, the carrier system is characterized by the fact that the recombinant protein, or the virus like particles, are obtained from prokaryotic or eucaryotic cells through the expression of the protein encoded by the appropriately modified RNA-2 gene of the FHV capsid protein.

20

#### Characteristics of the carrier particle.

##### Flock House Virus (FHV).

FHV is a non-enveloped icosahedral insect virus with a bipartite RNA genome 25 and belonging to the Nodaviridae family. These viruses are among the smallest and simplest known. The FHV genome consists of two single stranded mRNA molecules (RNA-1 with 3.1 kb and RNA-2 with 1.4 kb), both encapsidated in the same particle. RNA-1 carries the information for the viral RNA-polymerase and RNA-2 codes for the coat precursor, alpha protein. Upon synthesis the coat precursor alpha is rapidly 30 assembled with both RNAs, whereby immature, virion-like particles (provirions) are formed. These are slowly processed to mature particles by autocatalytic cleavages [12].

X-ray diffraction studies have shown the structure of the viral particles at 3.0 Angstrom resolution [13] (see fig 1). The virion has 60 icosahedral, asymmetric units each consisting of three quasiequivalent protomers forming a protein shell around the inner RNA genome [14]. The protomers consist of 1) a basic, crystallographically disordered aminotermminus, 2) a Beta-barrel structure, 3) an outer protrusion composed predominantly of Beta sheets and formed by three large insertions between the strands of the Beta-barrel, and 4) a carboxyterminal domain composed of two distorted helices lying inside the shell. The external zone of the virion, which is the least conserved, has many sequence differences which essentially contain all the deletions and insertions of the 10 different strains [12]. The variations in the loops, directed outwards from the segments of the Beta-barrel structure, define serologically distinct viral strains.

These loops were selected as the regions to be manipulated for the insertion of the foreign epitopes. The positions for these insertions ( L1, L2, L3, I1, I2, I3,) are given by the following amino acid regions of the RNA 2 gene:

15	Loop L1	amino acids 195-219
	Loop L2	amino acids 263-277
	Loop L3	amino acids 129-138
	Loop I1	amino acids 107-110
	Loop I2	amino acids 152-165
20	Loop I3	amino acids 304-310

In fig. 2, showing the DNA sequence of FHV RNA-2 and the corresponding amino acid sequence, the individual loop regions are accentuated.

Fig. 3 shows the full restriction map of the DNA sequence of FHV RNA-2.

25 Fig. 4 represents the number of cutting sites of the endonucleases.  
Fig. 5 shows all sites in which the endonucleases cut FHV RNA-2.  
Fig. 6 is a graphic representation of the unique cutting sites of the endonucleases.  
FHV grows vigorously in cultured cells and produces yields of 20% of the total cell protein [14]. In addition, FHV grows well in several Lepidopteran larvae. The viruses 30 of this family show a considerable resistance to inactivation by heat, detergents and other denaturants [14]. It was shown recently [15] that the expression of the capsid protein

FHV RNA-2 in insect cells via a recombinant baculovirus produces virion like particles (VLP) similar to authentic virions. It was shown by the present inventors that this can also be achieved by the expression of a modified gene carrying insertions for the expression of foreign amino acids within the capsomer structure. The VLPs generated by this procedure in insect cells are mature particles since the precursor protein, which is present in provirions, is cleaved. This system allows the production of 1 - 2 mg of purified synthetic virions (VLPs) in 50 ml of cultured cells [16].

Another method for the production of particles carrying foreign epitopes is by recovery of infectious virions after cotransfection of the genomic RNA-2 (obtained by in 10 vitro transcription of modified cDNAs) with purified RNA-1 [17]. This is only valid for genomes which carry alterations that do not change the replicative cycle or the assembly of the virus. The RNA-1 can be purified by several cycles of autonomous replication in DM-1 cells (*Drosophila Melanogaster*) taking advantage of the fact that RNA-1 behaves as an autonomous replicon in transfected cells [18].

15

**Detailed description of the construction of recombinant baculovirus carrying the wild type or modified capsomer gene.**

FHV was grown in DM-1 cells and purified in sucrose and CsCl gradients as described in [19]. The genomic RNA was extracted from the purified virions by treatment with proteinase K and by phenol-chloroform extraction. A single stranded cDNA was made with reverse transcriptase using 20 bases long oligonucleotides complementary to the 3' end sequence [12] (see fig 2). A double stranded cDNA was made with standard PCR techniques [20] amplifying the single stranded cDNA using a 20 bases long oligonucleotide, complementary to the 5'-end of the RNA-2, together with the first primer. Both primers carried extra bases coding for selected restriction enzyme sites (Bam-HI site for the 5'-end and Xba-I site for the 3'-end). After the PCR amplification, the double stranded DNA was gel-purified and ligated to pUC18 (Sma I site). For the in vitro transcription of RNA-2 the corresponding cDNA was inserted into the plasmid 30 pBluescript SKII (Stratagene) under the control of phage T7 polymerase. Examples of the above mentioned modifications of FHV RNA-2 are shown in table 1.

Table 1

Position	Restriction Sites	Comments																
L 1	Kpn I  (after mutagenesis)	<p>a) Aminoacids 205 to 209 (ATDPA) deleted from the original sequence.</p> <p>b) Val 204 mutagenized to Gly (GTT to GGT) to create Kpn I site:</p> <table style="margin-left: auto; margin-right: auto;"> <tr> <td>G204</td> <td>T205</td> </tr> <tr> <td>GGT</td> <td>ACC</td> </tr> <tr> <td>CCA</td> <td>TGG</td> </tr> </table> <p>c) After oligo insertion, GT duplicates</p>	G204	T205	GGT	ACC	CCA	TGG										
G204	T205																	
GGT	ACC																	
CCA	TGG																	
L 2	Pst I  (after mutagenesis)	<p>a) Aminoacids 270 to 273 (GSTG) deleted from the original sequence.</p> <p>b) Mutagenesis of the codon usage in L269 (CAG to CTG) and Q274 (CAA to CAG) to generate Pst I site:</p> <table style="margin-left: auto; margin-right: auto;"> <tr> <td>L269</td> <td>Q274</td> </tr> <tr> <td>CTG</td> <td>CAG</td> </tr> <tr> <td>GAC</td> <td>GTC</td> </tr> </table> <p>c) After oligo insertion, LQ duplicates.</p>	L269	Q274	CTG	CAG	GAC	GTC										
L269	Q274																	
CTG	CAG																	
GAC	GTC																	
L 3	Nhe I Spe I  (original)	<p>a) Aminoacids 128 to 134 (VPAGTTFP) deleted after doble digestion with Nhe I-Spe I:</p> <table style="margin-left: auto; margin-right: auto;"> <tr> <td>A126</td> <td>S127</td> <td></td> <td>T135</td> <td>S136</td> </tr> <tr> <td>NheI</td> <td>GCT</td> <td>AGC</td> <td>SpeI</td> <td>ACT</td> <td>AGT</td> </tr> <tr> <td>CGA</td> <td>TCG</td> <td></td> <td>TGA</td> <td>TCA</td> </tr> </table> <p>b) S127 and T135 are regenerated after oligo insertion. There are no duplications of aa.</p>	A126	S127		T135	S136	NheI	GCT	AGC	SpeI	ACT	AGT	CGA	TCG		TGA	TCA
A126	S127		T135	S136														
NheI	GCT	AGC	SpeI	ACT	AGT													
CGA	TCG		TGA	TCA														
I 3	Bsu36 I  (original)	<p>a) There is no loss of aa in the original sequence.</p> <p>b) Oligo insertion duplicates aa P304 and E305</p> <table style="margin-left: auto; margin-right: auto;"> <tr> <td>P304</td> <td>E305</td> <td>G</td> </tr> <tr> <td>CCT</td> <td>GAG</td> <td>G</td> </tr> <tr> <td>GGA</td> <td>CTC</td> <td>C</td> </tr> </table>	P304	E305	G	CCT	GAG	G	GGA	CTC	C							
P304	E305	G																
CCT	GAG	G																
GGA	CTC	C																
I 2	BamH I  (after mutagenesis)	<p>a) Aminoacids 154 and 155 (TT) are deleted after mutagenesis.</p> <p>b) Change of codon usage in S156 (TCA to TCC) to generate BamH I site:</p> <table style="margin-left: auto; margin-right: auto;"> <tr> <td>G153</td> <td>S156</td> </tr> <tr> <td>GGA</td> <td>TCC</td> </tr> <tr> <td>CCT</td> <td>AGG</td> </tr> </table> <p>c) GS duplicates after oligo insertion.</p>	G153	S156	GGA	TCC	CCT	AGG										
G153	S156																	
GGA	TCC																	
CCT	AGG																	
I 1	Bsu36 I  (after mutagenesis)	<p>a) Mutagenesis of G108 and Q109 to generate Bsu36 I site:</p> <table style="margin-left: auto; margin-right: auto;"> <tr> <td>G108</td> <td>Q109</td> </tr> <tr> <td>GGA</td> <td>CAG</td> </tr> <tr> <td>to: P108</td> <td>E109</td> </tr> <tr> <td>CCT</td> <td>GAG</td> </tr> <tr> <td colspan="2">G (cf D110)</td> </tr> </table>	G108	Q109	GGA	CAG	to: P108	E109	CCT	GAG	G (cf D110)							
G108	Q109																	
GGA	CAG																	
to: P108	E109																	
CCT	GAG																	
G (cf D110)																		

The cDNA of RNA-2 was also inserted into the vector pVL-1393 (Invitrogen) (Bam-HI/Xba-I sites). In this vector the gene is placed under the control of the polyhedrin promotor and flanked by sequences of the *Autographa californica Nuclear Polyhedrosis Virus* (AcNPV) which allow *in vivo* production of recombinant virus after cotransfection with AcNPV genomic DNA.

#### Introduction of foreign sequences in the cDNA of RNA-2

10 Small insertions/deletions in the sequences of RNA-2 were carried out by the PCR technique [20]. The epitope specific sequences were inserted into one or more of the selected sites either using restriction enzyme sites (when available) or by the PCR technique. The stereo diagram of the FHV capsid protein precursor in fig. 7 shows the sites where the specific HIV-1 sequences "IGPGRAF" were inserted. Those amino acids 15 were inserted into the positions L1, L2, L3, and I2, whereas the aminoacids "IGPGRAFE" were inserted into position I3. In all positions, except in position I3, certain amino acids were deleted: In position L1 amino acids 205 - 209 were deleted and aa 204 was mutated to create Kpn I site; in position L2 amino acids 270 - 273 were deleted and aa 269 and 274 were mutated to create Pst I site; in position L3 amino acids 20 128 - 134 were deleted after digestion with Nhe I-Spe I. In position I2 amino acids 154 - 155 were deleted. See table 1.

Examples of the insertion of foreign sequences into the recombinant FHV capsomer are listed in table 2.

25

30

TABLE 2

**Examples of foreign sequences included in FHV recombinant capsomer**

Sequences	Sites	Amino acids sequence and their characteristics	Expressed in
HBV-PreS1	I3	MGTNLNSVPNPPAFGANST- -NPDWDFNPAGGMQWNSTAL Tcell epitope. Receptor binding site.	E.coli
HBV-PreS2	I3	MQWNNSTALDPRVRGL B cell epitope	E.coli
HBV-S	L1,L2,L3 I2,I3	CTTPAQGNNSMFPSCCCTKPTDGNC B cell epitope	E.coli Baculovirus
HCV-core	L1,L2,L3 I2,I3	1. TNPKPQRKTKRNTNRRPQD 2. VKFPGGGQIVGGVYLLPRR B cell epitopes.	E.coli Baculovirus
HIV-1 gp120	L1,L2,L3 I2,I3	IQRGPGRFAF (IIIB) IGPGRAF (MN) FGPGQAL (Mal) IGPGRTL (NY5) KGPGRVI (RF) IGLGQAL (Z2) V3 loop.B cell epitope. Neutralizing epitope	E.coli Baculovirus
HIV-1 gp120	L1,L2,L3 I2,I3	1.GKAMYAPPI 2. NMWQE(K)VGKA (C4).B cell epitope. Neutralizing epitope	E.coli Baculovirus
HIV-1 gp41	L1,L2,L3 I2,I3	ELDKWAS B cell epitope Neutralizing epitope	E.coli Baculovirus
HIV-1 gp41	L1,L2,L3 I2,I3	IEEEGGERDRDR B cell epitope Neutralizing epitope	E.coli Baculovirus

Production of recombinant Baculovirus carrying the RNA-2 gene:

The cDNA of RNA-2 (wild-type or after genetic manipulation) was inserted into 5 the transfer vector pVL-1393 under a polyhedrin promotor (sites Bam HI and XbaI of the polylinker). This pUC9 based vector carries a segment of AcNPV in the sequence flanking its polylinker and allows the transfer of the foreign gene to a baculovirus genome after in vivo recombination (see fig. 8). Insect cells (*Spodoptera Frugiperda* SF-21 cells) were co-transfected (LipoFectin) with linear genomic DNA (non-viable) of 10 AcNPV (BaculoGold from PharMingen) and with the transfer vector carrying the FHV gene. After 4 days the virus progeny was harvested and titered. Thereafter, several recombinant viruses were plaque purified (3 to 4 times from well isolated plaques). These recombinants were denominated AcNPV-FHV. In some cases the VLPs can tolerate the insertion of up to 20 amino acids without alteration of the assembly process. In other 15 cases, where the insertions prevented the formation of VLPs, this could be circumvented by coinfection with both the wild-type and the modified baculovirus. Thereby, mosaic VLPs were generated carrying both types of capsomer structures.

Production and purification of VLPs from insect cells infected with AcNPV-FHV.

20

In order to obtain purified antigens for immunological antigen studies, Sf-21 cells (in suspension or as monolayer) were infected with recombinant baculoviruses at a multiplicity of infection of 10. Two to three days after the infection 0.5% nonidet P-40 and 0.1% Beta Mercaptoethanol (2-ME) were added to the medium. After 15 minutes on 25 ice, the cell debris were removed by centrifugation for 10 minutes at 12000 g. The VLPs in the supernatant were pelleted through a 30 wt/wt % sucrose cushion (50 mM HEPES, 0.1% 2-ME) at 40.000 rpm in an SW41 rotor for 3 hours at 4 C. The pellet was resuspended in 50 mM HEPES, 0.1% 2-ME and laid on a 10 ml 5-20 wt/wt % continuous sucrose gradient in the same buffer. The particles were sedimented in an 30 SW41 rotor at 40.000 rpm for 1 hour at 11 C. The fractions of the gradient were collected from the bottom and aliquots of each fraction were run on a 10% SDS

poyacrylamid gel in order to localize the particle peak. The fractions containing the VLPs were pooled, pelleted by centrifugation and resuspended in the same buffer. The protein content of these preparations was determined by Micro BCA Protein Assay Reagent (Pierce).

5

Recovery of modified FHV containing exogenous sequences.

Live, recombinant FHV viruses can be recovered when the heterologous amino acid sequences which are inserted into the capsomer do not alter the virion assembly process. 10 The recovery was carried out by co-transfection of DM-1 cells with *in vitro* made transcripts of modified RNA-2 and authentic RNA-1 purified of RNA-2 by multiple transfection passages at the limiting dilution as described by Ball [18].

Example 1:

15 Production of VLPs carrying the HIV-1 (Human Immunodeficiency Virus, type 1) specific sequence IGPGRAF:

Several domains of the HIV-1 gp-120 can induce the production of neutralizing antibodies. One of them is the hypervariable region 3 (V-3 loop). This is a linear, 20 immunodominant epitope known as the "Principal Neutralizing Determinant" (PND) [21, 22]. Although the entire domain varies greatly among different isolates, it was recently found that, maybe due to conformation restraints, the amino acid sequence on the tip of the loop is well conserved. Sequence data from 245 isolates from the USA showed that the V3 loop sequence "GPGRAF" was present in more than 60% of the isolates [23]. In 25 addition, it was found that animals immunized with peptides containing this sequence produced sera which could neutralize several diverging isolates, although with a low titer [24]. This sequence was inserted into five different positions on the surface of the FHV structure and in some cases in two sites of the same molecule. The positions selected were the outwards directed loops mentioned above (see FHV structure fig.1). In one case 30 (position I3) the foreign sequence was introduced directly as an insert in the original sequence of RNA-2. In order to obtain this, the cDNA coding for the FHV capsidprotein

was digested with *Bsu* 36I (cuts the DNA at nucleotide 934), and a synthetic oligonucleotide was inserted coding for the HIV-1 specific sequence. As a consequence of this procedure an additional glutamic acid was inserted at the carboxyterminus of the HIV-1 sequence. The structures of all these recombinant proteins are shown in fig. 7.

5 Fig. 9 shows the proteins induced in Sf-21 cells after infection with the recombinant baculovirus carrying the HIV-1 epitopes in the positions shown in fig. 7. Cells infected with these recombinants, mock infected cells and cells infected with baculoviruses without inserts were lysed and analyzed in a 10% polyacrylamide gel. Coomassie staining of the gel showed, in the lysates from cells infected with recombinant 10 viruses, the presence of bands with a molecular weight similar to the expected molecular weight for FHV capsomer precursor protein (alpha protein) or its cleavage product. These bands were not present in the case of lysates from cells infected with baculovirus without the insert (AcNPV-RP6). Western blots from similar gels, analyzed with rabbit hyperimmune anti-FHV serum, confirmed the identity of the chimeric proteins. In 15 addition to the alpha precursor, its cleavage product (the mature beta protein) was seen in all cases. This probably indicates that the modified capsomers are still capable of assembling and autocleaving. However, in some cases the percentage of mature protein seemed to be low (e.g. L3; I3; I2), probably indicating that the presence of the insert affects the autocleavage process. When a similar blot was analyzed, either using sera 20 from HIV-1 positive patients or HIV-1 specific human monoclonal antibodies, a quite different pattern of recognition developed. The patients' sera mainly recognized the epitope in the L2 position or in those combinations where this position was used. On the contrary, the monoclonal antibodies strongly recognized the position L1 or combinations derived from that position. Position L3 was also extensively recognized by patients' sera 25 though consistently less than L2. The other positions were barely detectable by these sera. On the other hand, certain human sera detected preferentially proteins carrying the inserts in the positions L3 or I3. This suggests a difference in the specificity of the individual immune response to the same sequence. However, until now the strongest signals were always obtained when the proteins carried the inserts in the positions L1 or L2. 30 Coomassie staining of the gels showed that the differences do not depend upon the amount of induced protein in the insect cells. This confirmed the hypothesis that the

antigenicity of the epitope is influenced by its localization. Until now the reason for the differences in the patterns of recognition in different patients could not be explained. Further investigations are now to be carried out to explain these data. For example concerning the origin of the infecting strain, the neutralization titer of the sera, the 5 differences in the idiotypic answers, the difference in the patients' prognosis, etc.

Purification of VLP-V3 from recombinant, baculovirus infected Sf-21 cells.

Fig.10 shows the sedimentation profile and the antigen composition of VLPs produced by three different baculovirus: AcNPV-FHV which expresses the unmodified FHV capsid protein; AcNPV-FHV-V3/L1 expressing the same protein yet carrying the HIV-1 epitope in position L1; and AcNPV-FHV-V3/L2 carrying the insert in position L2. See fig. 7 for details on insert locations. In all cases it was found that the particulate material, obtained as described above, migrated to the same position in the gradient as the FHV particles. The particulate nature of these components was further confirmed by electron microscopy. Aliquots from each peak were run on a polyacrylamide gel and probed with HIV-1 positive serum after transfer to nitrocellulose paper. In all cases the detected proteins migrated to the same position as did the FHV capsid protein or its precursor. In the case of wild-type or L1-derived particles the main band corresponded to the mature protein, whereas in L2-derived particles a large quantity of immature protein (alpha protein) was present in addition. However, there seemed to be an increase of mature protein in VLPs when these were compared with the input material prior to the purification. Similar results were also obtained after analysis of the products of those recombinant baculoviruses expressing the FHV capsid proteins with the inserts in the other positions described above. The only differences found were the yield of particulate material and the percentage of VLPs carrying immature protein in that material.

The experiments were carried out as follows. Four days after the infection the cells and the medium were processed as previously described and the presence of the particles was analyzed by sucrose density gradient sedimentation (SW50.1 rotor at 45000 rpm for 30 minutes at 20 degrees C). The fractions were collected from the bottom of the tubes. In order to detect the distribution of the FHV along the gradient, aliquots of each

fraction were tested for FHV reactivity by means of an ELISA assay. The distribution of HIV-1 specific reactivity along the gradient was measured through western blotting of samples from the peak fractions. The western blots were probed with HIV-1 positive sera and the resulting bands are shown at the bottom of each graph. In fig.10 graph a) shows 5 the reactivity of AcNPV-FHV-derived particles, b) shows particles derived from AcNPV-FHV-V3-L1 and c) shows particles derived from AcNPV-FHV-V3-L2. The arrows indicate the migration of FHV run in a parallel gradient.

#### Immunogenicity of chimeric VLP-V3 particles

10

In order to determine whether the seven HIV-amino acids inserted into the VLP structure were capable of inducing an immune response, three groups of guinea pigs, each consisting of three animals, were immunized with purified VLPs carrying the HIV-1 insert as described in the following. The first group of animals was inoculated with the 15 insert in position L3, the second group with the insert in position I3 and the third group was inoculated with the insert in both positions L3 and I3. All three groups were immunized subcutaneously with 500 microlitres PBS containing 50 microgrammes of the respective VLP preparations. For the first immunizations on day 0 the antigens were formulated in complete Freund's adjuvant (CFA). For the boosters on days 14 and 28 the 20 same amount of antigen was formulated in Freund's incomplete adjuvant (IFA). Blood was taken from each animal 35 days after the first inoculation by cardiac puncture. Sera from the immunized animals were tested for specific anti-V3 and anti-FHV antibodies in an ELISA test. The data represent reciprocal dilutions at OD 492. For the anti-FHV titer the ELISA plates were coated with CsCl-purified viruses (200 nanogrammes per well). 25 The titers against the HIV-1 inserts were analyzed on plates coated with recombinant gp120 (ABT-Baculovirus produced, 100 ng per well) as a capture antigen. The data in fig. 11 show that these preparations had elicited a good antibody response specific for the V3 sequence. As shown by this test, no major differences existed among the various constructs. However, the rest of the positions are yet to be analyzed and an evaluation is 30 to be made of the differences in the affinity shown to the native gp-120 by the immune sera, a parameter known to be associated with their neutralizing capacity.

**Example 2:****Hepatitis C Virus (HCV)**

5

The transfusion induced Hepatitis, which can neither be attributed to Hepatitis A virus nor to Hepatitis B virus (NANBH), belongs to the main group of transfusion transmitted diseases [25]. The cloning and expression of HCV has allowed the development of antibody screening immunoassays for the detection of HCV infections, 10 using as solid phase antigen a fusion polypeptide expressed through recombinant yeast. Initial studies using this protein confirmed that HCV was the predominant agent of NANBH. However, these and subsequent studies demonstrated a series of shortcomings with this serological test due to low sensitivity and specificity. The tests currently used are mostly based on the detection of antibodies against the non-structural proteins 15 NS3-NS4 which, however, do not appear in infected patients until the disease is in an advanced state (4-6 months after the onset of the Hepatitis). Later, it was demonstrated that most immunodominant epitopes are located within the aminoterminal parts of the core protein [25, 26, 27] and that antibodies against these epitopes appear early after the infection. This was shown either by using recombinant HCV-core protein produced in 20 bacteria or baculovirus, or by using synthetic peptides corresponding to these sequences. Moreover, the HCV-core protein is considered as the most significant single antigen for the detection of antibodies in infected patients. Among all positive samples 80-85 % are found to be core positive and in most of them this was the only antigen recognized.

25

**Production of FHV-VLPs carrying an HCV-core epitope.**

With reference to these considerations, the epitopes of the HCV core protein were tested in the molecular presentation system of the invention here reported. A 20 amino acids long sequence was selected corresponding to the amino acids 20-40 in the original 30 sequence about which it had already been shown that they were very effective for diagnostic purposes [26]. The 20 amino acids long epitope was inserted in the I3 position

on the Bsu-36I site of the RNA-2 gene contained in pVL-1393. The recombinant Baculovirus was produced and purified as described. The resulting recombinant was denominated AcNPV-HCVc. Fig. 12 shows the expression of hybrid FHV-HCV proteins through the recombinant baculovirus AcNPV-HCVc. The chimeric capsomer was produced through the recombinant baculovirus as follows. Sf-21 cells were infected with recombinant baculovirus AcNPC-HCVc (lane 3, 6, and 7), with a baculovirus carrying an unmodified FHV capsid protein AcNPV-FHV (lane 2 and 4) and with a polyhedrin-minus baculovirus carrying no insert AcNPV-RP6 (lane 1), respectively. The whole cell extracts were run in a 10% SDS-PAGE gel. Purified FHV was included as a 10 marker (lane 5). Lanes 1-3 were stained with Coomassie blue. After the running, the proteins in lanes 4-7 were blotted on Nitrocellulose paper. After staining with Poinceau red, paper strips corresponding to each well were cut out and probed with a specific serum. Lanes 4 and 6 were probed with serum from a patient who was core positive in a RIBA-II test. Lanes 5 and 7 were probed with rabbit-anti-FHV serum.

15 The insect cells were infected with the recombinant virus and four days after the infection the cells were lysed and analyzed on a 10% SDS-PAGE gel. After the running, the gel was stained with Coomassie brilliant blue. The introduction of HCV-sequences apparently had no influence on the protein production. However, all the detected protein migrated with the molecular weight of the precursor (alpha protein). This indicates that 20 the maturation process is somewhat impaired by the sequence alteration. In order to confirm the identity of this protein, the lysates of the infected cells were run on a similar gel, transferred to Nitrocellulose paper and probed with specific antisera. As expected, the protein reacted strongly with the specific rabbit-anti-FHV antiserum (dilution 1:2000) as well as with HCV positive human serum (dilution 1:200).

25 Fig.13 shows the FHV reactivity (measured in an ELISA test) after sucrose sedimentation of VLPs produced by infection of Sf-21 cells with the recombinant baculovirus AcNPV-HCVc. The running conditions were identical to those described in connection with fig.10. Aliquots from each peak fraction were western blotted and probed with HCV positive human sera. A photo of the developed Western bands is 30 inserted at the bottom of the graph. The arrow indicates the position of FHV run in a parallel gradient.

As in the case of the particles carrying HIV-1 specific sequences, the particles migrated somewhat slower than the wild-type FHV particles. Western blots of aliquots from the peak reacted with HCV-positive sera. This indicates that the unprocessed protein is not impaired in its ability to autoassemble into a particulate structure.

5 To asses the capability of the antigen to detect specific antibodies, purified VLPs were used for the ELISA test. Wells of ELISA plates (Nunc) were coated with 100 microlitres of purified VLPs diluted in PBS buffer (100 nanogrammes per well). After blocking with PBS containing 5% BSA, 100 microlitres of serum dilution were added to each well and the plate was incubated for two hours at room temperature. The bound 10 antibodies were detected by a second incubation with a horse radish peroxidase conjugate of the IgG fraction of goat anti-human immunoglobulin for one hour at room temperature. The enzyme activity was measured using o-phenylenediamine as a substrate. The absorbance of each well was measured at  $A = 490$  nanometer. To test the sensitivity of this antigen, 100 sera, known to be core positive in a commercial test (RIBA II-Chiron 15 Corp.), were analyzed. Almost 85% of the samples gave titres higher as 1:1000 which indicates a very good sensitivity when detecting anti-core antibodies. These results demonstrated that these 20 amino acids from the HCV core sequence represent a very reliable antigen for the detection of HCV infections, when introduced in the carrier system of the present invention.

20 The results are represented in the block diagram in fig. 14.

#### Comparison of a VLP-based ELISA test with current, commercially available tests.

In order to test the sensitivity of this ELISA assay, a collection of serially drawn 25 blood samples from infected patients, encompassing the period of seroconversion, were analyzed for specific HCV core antibodies. At that stage, all patients already have a high level of the specific liver enzyme Alanine aminotransferase (ALT). In four out of 50 patients which were analyzed (see fig. 15) the test subject of this invention showed seroconversion earlier than in the currently available testkits (RIBA II). These results 30 show that the antigen is extremely suitable for detection of contaminated samples in blood banks. The serially drawn blood samples from selected patients, taken for the RIBA-II

test before the seroconversion, were analyzed by using plates coated with VLPs carrying an HCV core-specific epitope. The serum dilution was 1:100 for RIBA-II as well as for the ELISA test of the invention here reported. The RIBA-II values are shown in the upper panel. The VLP-based ELISA test values are represented in the diagram as circles, the 5 ALT values as a vertical line.

A comparison between antibody detection by VLPs, carrying HCV core sequences,  
and antibody detection by the free HCV peptide.

10 It has been shown that short peptides are very efficient when used as capture antigens for detection of specific antibodies in human as well as animal sera especially in the form of branched peptides [29]. It has also been reported that they react better than the corresponding recombinant antigens [30]. In transfusion induced Hepatitis-C cases it was established that by using peptides as capture antigens, positive sera could be detected  
15 as early as one month after the first transfusion. This coincides with the first increase in the specific liver enzymes and would make short amino acid sequences a useful marker for detecting acute specific HCV infections [27]. For this reason it was decided to compare in a dot-blot assay the HCV specific antigens described in the invention at issue with the corresponding free peptides (HCc-2p), with a peptide encompassing the first 20  
20 amino acids of the core (HCc-1p), and with peptides corresponding to other HCV proteins (NS peptides), respectively.

Fig. 16 shows a photograph of these dot-blots which were carried out as follows. Aliquots of 10 microlitres of purified VLPs (5 microgrammes per ml), carrying an insert of 20 amino acids from the core of HCV, and solutions containing peptides representing 25 different areas of the HCV genome (100 microgrammes per ml) were blotted on nitrocellulose paper. After blocking with 5% fat free and dry milk, each strip was incubated with a 1:100 dilution of human sera. After washing, each filter was incubated with anti-human antibodies conjugated to horse radish peroxidase (Dako, dilution 1:5000), and finally incubated with diamino benzidine (DAB). Lanes 1 - 6 show patients' 30 sera. Lane NC shows the negative control serum.

17

As can be seen, already very low levels of antigen (50 nanogrammes corresponding to 2.5 nanogrammes of the specific HCV peptide) are strong enough, in the form of VLPs, to elicit a good signal with a positive sample. The corresponding free peptide (HCc-2p) gave only a very weak signal although it recognized the same number of positive samples. In this case, the amount of antigen loaded onto the nitrocellulose paper was 400 times higher as in the case of the VLPs, based on a molar ratio. The peptide corresponding to the first 20 amino acids (HCc-1p) gave stronger signals, but failed to detect one positive sample and gave an indeterminate result with another positive sample. Peptides which corresponded to other HCV proteins and which were designed on 10 the basis of published results [31] are far less efficient for detection of HCV positive sera.

15

20

25

30

## CLAIMS:

1. A molecular presentation system in which viral proteins are used as carriers for 5 specific amino acid sequences and which is characterized by the fact that the viral protein is derived from small insect viruses of known 3-dimensional structure and amino acid sequence - preferentially from FHV - in which heterologous amino acid sequences, such as epitopes, are inserted into the outwards directed loops of the capsid protein.

2. A system according to claim 1, characterized by the fact that a recombinant 10 protein, or virus like particle, is used as viral protein whereby this recombinant protein, or virus like particle, is obtained from prokaryotic or eukaryotic cells through the expression of the protein encoded by the appropriately modified RNA-2 gene of the FHV capsid protein, as shown in figure 2.

3. A system according to claim 2, characterized by the fact that the heterologous 15 amino acid sequences are inserted into one or more regions of the outwards directed loops of the FHV capsid protein, encoded by the RNA-2 gene, in which the loops denoted L1,L2,L3,I1,I2,I3 are chosen.

4. A system according to claim 3, characterized by the fact that the regions of the 20 loops selected for the insertion of the heterologous amino acid sequences are defined by the following amino acid sequence regions of the FHV Capsid protein encoded by the RNA-2 gen:

- Loop L1 amino acid sequence region 195 - 219  
Loop L2 amino acid sequence region 263 - 277  
25 Loop L3 amino acid sequence region 129 - 138  
Loop I1 amino acid sequence region 107 - 110  
Loop I2 amino acid sequence region 152 - 165  
Loop I3 amino acid sequence region 304 - 310

30 5. A process for the production of a molecular presentation system according to claims 3 or 4 characterized by the fact that the RNA-2 gene of the FHV capsid protein, in

19

the regions corresponding to loops L1, L2, L3, I1, I2, I3, is modified by deletions and/or mutagenesis and/or insertions in order to create cut sites for restriction enzymes in the said loop regions that can be used for the inserts which encode the heterologous amino acid sequences.

5        6. Application of a molecular presentation system according to one of the claims 1 to 4 for therapeutic, diagnostic and immunization purposes.

10

15

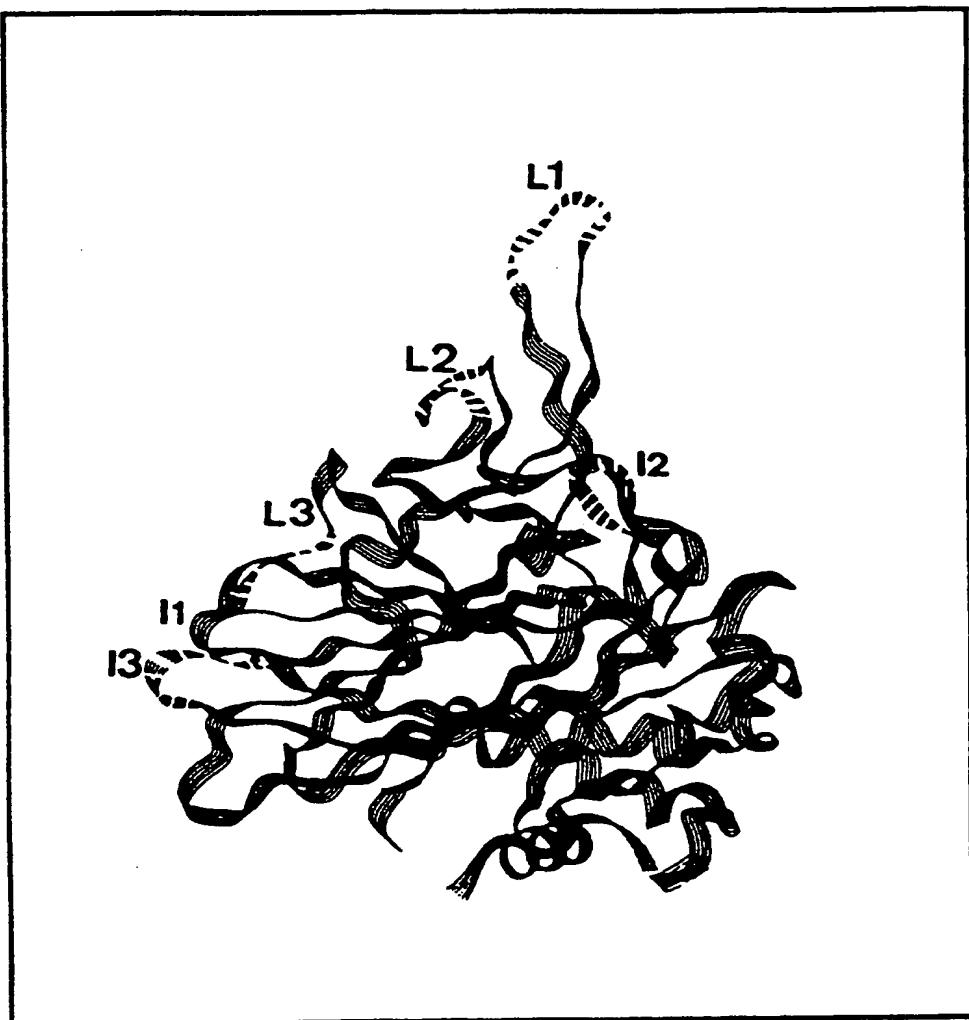
20

25

30

1/19

**FIG.1** Crystallographic representation of the outward directed loops of the FHV Capsid protein precursor with 3 Å resolution, showing the positions of insertions of the foreign genes (L1, L2, L3, I1, I2, I3).



2/19

23 53  
atggtaataacaacagaccaagacgtcaacgagctcaacgcgttgcgtcacaacaacc  
M V N N N R P R R Q R A Q R V V V T T T

83 113  
caaacagcgccctgttccacagcaaaacgtgccacgtaatggtagacgccacgtaatcg  
Q T A P V P Q Q N V P R N G R R R R N R

143 173  
acgaggcgtaatcgccgacgtgtgcgcggaatgaacatggcggcgctaaccagattaagt  
T R R N R R R V R G M N M A A L T R L S

203 233  
caacctggttggcgttctcaaattgtgcattgcaccacctgacttcaacaccgacccc  
Q P G L A F L K C A F A P P D F N T D P

263 293  
ggtaagggaaatacctgatagattgaaggcaaagtggtcagccgaaaggatgtcctcaat  
G K G I P D R F E G K V V S R K D V L N

323 353  
caatctatcagttactgccggacaggacactttatactcatgcacctaccccccgg  
Q S I S F T A G Q D T F I L I A P T P G

383 413  
gtcgccctactggagtgctagcgccccctgtggactttctacttagtgcgactacgttt  
V A Y W S A S V P A G T F P T S A T T F

443 473  
aaccccgtaattatccgggtttacatcgatgtcgaaacaacttcaacatctagggcc  
N P V N Y P G F T S M F G T T S T S R S

503 533  
gatcagggtgcctcattcaggtacgcttccatgaacgtggatttacccaacgtcgAAC  
D Q V S S F R Y A S M N V G I Y P T S N

3/19

563 593  
ttgatgcagttgccggaagcataactgttggaaatgccctgtaaagctgagtactgtg  
L M Q F A G S I T V W K C P V K L S T V

623 653  
caattcccggttgcaacagatccagccaccaggctcgctagttcataactcttgggtta  
Q F P V A T D P A T S S L V H T L V G L

683 713  
gatggtgttctagcggtggggcctgacaacttctgagtcattcatcaaaggagtgtt  
D G V L A V G P D N F S E S F I K G V F

743 773  
tcacagtcggcttctaactggcctgactttgaattcaatgacatattggagggtatccag  
S Q S A C N E P D F E F N D I L E G I Q

803 833  
acattgccacctgctaatgtgtcccttgggttctacgggtcaacctttaccatggactca  
T L P P A N V S L G S T G Q P F T M D S

863 893  
ggagcagaagccaccagtggagttagtcggatggggcaatatggacacgattgtcatccgt  
G A E A T S G V V G W G N M D T I V I R

923 953  
gtctcgccccctgagggcgcagttactctgcataactcaaggcatggcctgcatttag  
V S A P E G A V N S A I L K A W S C I E

983 1013  
tatcgaccaaattccaaacgccatgttataccaattcggccatgattgcctcctcgat  
Y R P N P N A M L Y Q F G H D S P P L D

1043 1073  
gaggtcgcgcttcaggaataccgtacggtgccagatcttgcgggtgcagtgatagcg  
E V A L Q E Y R T V A R S L P V A V I A

4/19

1103 1133  
gcccaaaatgcataatgtggagagagtgaaatccatcataaaatcctccctggctgct  
A Q N A S M W E R V K S I I K S S L A A

1163 1193  
gcaagcaacattcccgccccatcggtgtcgccgcaagtggatttagtggactgtcagcc  
A S N I P G P I G V A A S G I S G L S A

1223 1253  
cttttgaaggattggctttagaagcatccggacgccaacctaacccggcaagtatcc  
L F E G F G F STOP

1283 1313  
gaacaatcgacatggccacaataagccaaatttgttgaagattaaagttagtgagcc

1343 1373  
cccttagcgcgaaaccggaatttatattccaaaccagtttaagtcaacagactaagg

## Restriction Endonucleases site usage

Aat II	1	Bsp1286I	2	Fse I	-	Pac I	-
Acc I	1	Bsp1407I	-	Fsp I	-	PaeR7 I	-
Acc65 I	-	BspD I	1	Gdi II	1	PflM I	1
Aci I	5	BspE I	1	Hae I	1	Ple I	3
Afl II	-	BspH I	-	Hae II	2	Pme I	-
Afl III	2	BspM I	1	Hae III	6	Pml I	-
Age I	-	BspW I	4	Hga I	2	Ppu10 I	1
Alu I	3	Bsr I	4	HgiA I	1	PpuM I	-
Alw I	1	BsrF I	1	Hha I	6	PshA I	-
AlwN I	1	BssH II	-	Hinc II	5	Psp1406I	-
Apa I	-	Bst1107I	-	Hind III	-	Pst I	-
ApaL I	-	BstB I	-	Hinf I	4	Pvu I	1
Apo I	2	BstE II	-	HinP I	6	Pvu II	-
Asc I	-	BstK I	8	Hpa I	1	Rsa I	4
Ase I	-	BstN I	2	Hpa II	11	Rsr II	-
Ava I	-	BstU I	4	Hph I	-	Sac I	1
Ava II	2	BstX I	1	Kas I	-	Sac II	-
Avr II	-	BstY I	2	Kpn I	-	Sal I	-
BamH I	-	Bsu36 I	1	Mae II	8	Sap I	-
Ban I	-	Cla I	1	Mae III	2	Sau3A I	4
Ban II	2	Csp6 I	4	Mbo I	4	Sau96 I	6
Bbe I	-	Dde I	6	Mbo II	1	Sca I	1
Bbs I	-	Dpn I	4	Mcr I	1	ScrF I	8
Bbv I	2	Dpn II	4	Mlu I	1	SfaN I	3
BceF I	-	Dra I	-	Mme I	1	Sfc I	-
Bcg I	1	Dra III	-	Mnl I	9	Sfi I	-
Bcl I	-	Drd I	2	Msc I	1	SgrA I	-
Bcn I	6	Dsa I	1	Mse I	8	Sma I	-
Bfa I	5	Dsa V	8	Msp I	11	SnaB I	-
Bgl I	-	Eae I	2	Mun I	-	Spe I	1
Bgl II	1	Eag I	-	Nae I	-	Sph I	-
Bpm I	1	Eam1105I	-	Nar I	-	Srf I	-
Bpu1102I	-	Ear I	-	Nci I	6	Sse8337I	-

6/19

Bsa I	-	Ecl136 I	1	Nco I	1	Ssp I	-
BsaA I	1	Eco47 III	-	Nde I	-	Stu I	-
BsaB I	1	Eco57 I	1	NgoM I	-	Sty I	2
BsaH I	3	EcoN I	1	Nhe I	1	Swa I	-
BsaJ I	5	EcoO109 I	1	Nla III	6	Taq I	4
BsaW I	2	EcoR I	1	Nla IV	2	Tfi I	1
Bsg I	-	EcoR II	2	Not I	-	Tth111 I	-
BsiE I	1	EcoR V	-	Nru I	-	Tth111II	3
BsiW I	1	Ehe I	-	Nsi I	1	Xba I	-
Bsl I	6	Esp3 I	-	Nsp I	-	Xcm I	-
Bsm I	-	Fau I	-	Nsp7524I	-	Xho I	-
BsmA I	1	Fnu4H I	5	NspB II	-	Xma I	-
Bsp120I	-	Fok I	4	NspC I	-	Xmn I	1

## Enzyme/Recognition sequence/n.sites/positions:

Aat II	gacgt/c	1	45
Acc I	gt/mkac	1	123
Alw I	ggatc 4/5	1	641
AlwN I	cagn3/ctg	1	87
Bcg I	cgan6tgc	1	558
Bgl II	a/gatct	1	1076
Bpm I	ctggag 16/14	1	391
BsaA I	yac/gtr	1	114
BsaB I	gatnn/nnatc	1	910
BsiE I	cgry/cg	1	1182
BsiW I	c/gtacg	1	1064
BsmA I	gtctc 1/5	1	923
BspD I	at/cgat	1	469
BspE I	t/ccgga	1	1252
BspM I	acctgc 4/8	1	811
BsrF I	r/ccggy	1	1084
BstX I	ccan5/ntgg	1	531
Bsu36 I	cc/tnagg	1	932
Cla I	at/cgat	1	469

7/19

Dsa I	c/crygg	1	852
Ecl136 I	gag/ctc	1	54
Eco57 I	ctgaag	16/14	1 1052
EcoN I	cctnn/n3agg	1	513
EcoO109 I	rg/gnccy	1	700
EcoR I	g/aattc	1	773
Gdi II	yggccg	-5/-1	1 1018
Hae I	wgg/ccw	1	1298
HgiA I	gwgcw/c	1	54
Hpa I	gtt/aac	1	944
Mbo II	gaaga	8/7	1 1323
Mcr I	c/grycg	1	1182
Mlu I	a/cgcgt	1	61
Mme I	tccrac	20/18	1 887
Msc I	tgg/cca	1	1298
Nco I	c/catgg	1	852
Nhe I	g/ctagc	1	398
Nsi I	atgca/t	1	1110
PflM I	ccan4/ntgg	1	113
Ppu10 I	a/tgcata	1	1110
Pvu I	cgcgt/cg	1	1182
Sac I	gagct/c	1	54
Sca I	agt/act	1	614
Spe I	a/ctagt	1	425
Tfi I	g/awtc	1	1025
Xmn I	gaann/nnttc	1	480
Afl III	a/crygt	2	61 160
Apo I	r/aatty	2	773 1360
Ava II	g/gwcc	2	498 969
Ban II	grgcy/c	2	54 1338
Bbv I	gcagc	8/12	2 1157 1160
BsaW I	w/ccggw	2	1252 1356
Bsp1286I	gdgch/c	2	54 1338
BstN I	cc/wgg	2	206 1153
BstY I	r/gatcy	2	640 1076

8/19

DrdI	gacn4/nngtc	2	39	905
Eae I	y/ggccr	2	1018	1298
EcoR II	/ccwgg	2	206	1153
Hae II	rgcgc/y	2	88	184
Hga I	gacgc	5/10	2	126 1256
Mae III	/gttnac	2	71	756
Nla IV	ggn/ncc	2	700	928
Sty I	c/cwwgg	2	826	852
Alu I	ag/ct	3	55	332 609
BsaH I	gr/cgyc	3	45	126 1256
Ple I	gagtc	4/5	381	719 857
SfaNI	gcatac	5/9	565	1112 1249
Tth111II	caarca	11/9	3	83 589 1164
BspWI	gcn5/nngc	4	103	1091 1103 1340
BsrI	actgg	1/-1	4	390 651 876 1376
BstU I	cg/cg	4	62	167 1048 1350
Csp6 I	g/tac	4	414	523 615 1065
Dpn I	ga/tc	4	503	641 1077 1183
Dpn II	/gatc	4	503	641 1077 1183
FokI	ggatg	9/13	4	310 890 916 1250
Hinf I	g/antc	4	381	719 857 1025
Mbo I	/gatc	4	503	641 1077 1183
Rsa I	gt/ac	4	414	523 615 1065
Sau3AI	/gatc	4	503	641 1077 1183
Taq I	t/cga	4	470	557 985 1038
Aci I	ccgc	-3/-1	5	168 182 695 1100 1194
Bfa I	c/tag		5	399 426 495 659 692
BsaJ I	c/cnngg		5	259 376 826 852 1152
Fnu4H I	gc/ngc		5	182 1100 1157 1160 1193
Hinc II	gty/rac		5	48 201 840 944 1385
Bcn I	ccs/gg	6	260	377 458 628 1175 1269
Bsl I	ccn5/nngg	6	113	259 260 371 513 919

9/19

Dde I	c/ttag	6	611 717 859 933 1345 1394
Hae III	gg/cc	6	702 928 1019 1102 1178 1299
Hha I	gcg/c	6	89 166 185 939 1049 1349
HinP I	g/cgc	6	89 166 185 939 1049 1349
Nci I	cc/sgg	6	260 377 458 628 1175 1269
Nla III	catg/	6	178 532 853 966 1003 1022
Sau96 I	g/gncc	6	498 701 928 969 1102 1178
BstK I	c/cngg	8	206 260 377 458 628 1153 1175 1269
Dsa V	/ccnngg	8	206 260 377 458 628 1153 1175 1269
Mae II	a/cgt	8	46 108 115 133 160 437 537 554
Mse I	t/taa	8	27 197 441 450 945 1143 1328 1381
ScrF I	cc/ngg	8	206 260 377 458 628 1153 1175 1269
MnlI	cctc	7/7 9	145 316 513 791 935 1031 1034 1043
1149			
Hpa II	c/cgg	11	261 342 378 458 576 629 1085 1176
1253 1269 1357			
Msp I	c/cgg	11	261 342 378 458 576 629 1085 1176
1253 1269 1357			

292 sites found

No Sites found for the following Restriction Endonucleases

Acc65I	g/gtacc	DraIII	cacn3/gtg	PmeI	gttt/aaac
AflII	c/ttaag	EagI	c/ggccg	PmlI	cac/gtg
AgeI	a/ccgg	Eam1105I	gacn3/nngtc	PpuMI	rg/gwccy
ApaI	gggcc/c	EarI	ctcttc 1/4	PshAI	gacnn/nngtc
ApaLI	g/tgcac	Eco47III	agc/gct	Psp1406I	aa/cgtt
AscI	gg/cgcgcc	EcoRV	gat/atc	PstI	ctgca/g
AseI	at/taat	EheI	ggc/gcc	PvuII	cag/ctg
AvaI	c/ycgrg	Esp3I	cgtctc 1/5	RsrII	cg/gwccg
AvrII	c/ctagg	FauI	cccgcc 4/6	SacII	ccgc/gg

10/19

BamHI	g/gatcc	FseI	ggccgg/cc	SalI	g/tcgac
BanI	g/gyrcc	FspI	tgc/gca	SapI	gcttttc 1/4
BbeI	ggcgc/c	HindIII	a/agctt	SfcI	c/tryag
BbsI	gaagac	2/6 HphI	ggtga 8/7	SfiI	ggccn4/nggcc
BceFI	acggc12/13	KasI	g/gcgcc	SgrAI	cr/ccggyg
BclI	t/gatca	KpnI	ggtac/c	SmaI	ccc/ggg
BglII	gccn4/nggc	MunI	c/aattg	SnaBI	tac/gta
Bpu1102I	gc/tnagc	NaeI	gcc/ggc	SphI	gcatg/c
BsaI	ggtctc1/5	NarI	gg/cgcc	SrfI	gccc/gggc
BsgI	gtgcag16/14	NdeI	ca/tatg	Sse8337I	cctgca/gg
BsmI	gaatgc	1/-1 NgoMI	g/ccggc	SspI	aat/att
Bsp120I	g/ggccc	NotI	gc/ggccgc	StuI	agg/cct
Bsp1407I	t/gtaca	NruI	tcg/cga	SwaI	attt/aaat
BspHI	t/catga	NspI	rcatg/y	Tth111I	gacn/nngtc
BssHII	g/cgcgc	Nsp7524I	r/catgy	XbaI	t/ctaga
Bst1107I	gta/tac	NspBII	cmg/ckg	XcmI	ccan5/n4tgg
BstBI	tt/cgaa	NspCI	rcatg/y	XhoI	c/tcgag
BstEII	g/gtnacc	PacI	ttaat/taa	XmaI	c/ccggg
DraI	ttt/aaa	PaeR7I	c/tcgag		

11 / 19

۶۷

123	Acc I	558	Bcg I	944	Hpa I	1110	Ppu10 I
114	BsaA I	531	BstX I	932	Bsu36 I	1182	Pvu I
113	PfM I	513	EcoN I	923	BsmA I	1182	Mcr I
87	AlwN I	480	Xmn I	910	BsAB I	1182	BsiE I
61	Mlu I	469	Cla I	887	Mme I	1110	Nsi I
54	Sac I	469	BspD I	852	Nco I	1076	Bgl II
54	HgiA I	469	Spe I	773	EcoR I	1025	Tth I
54	Ecl136 I	398	Nhe I	700	EcoO109 I	1018	Gdi II
45	Aat II	391	Bpm I	641	Alw I	1064	BsiW I
				614	Sca I	1052	Eco57 I
				811	BspM I	1323	Mbo II

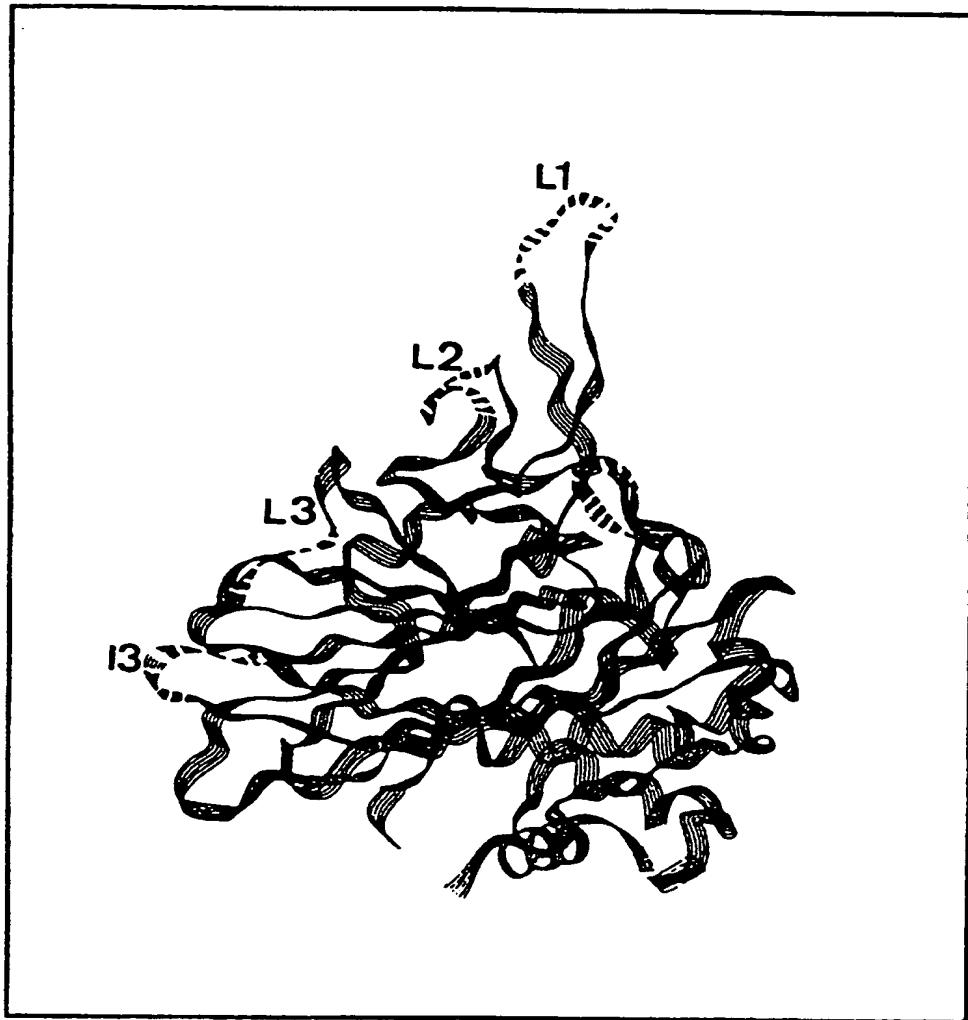
FHV RNA2 1400 base pairs Unique Sites

1400 base pairs

**SUBSTITUTE SHEET (RULE 26)**

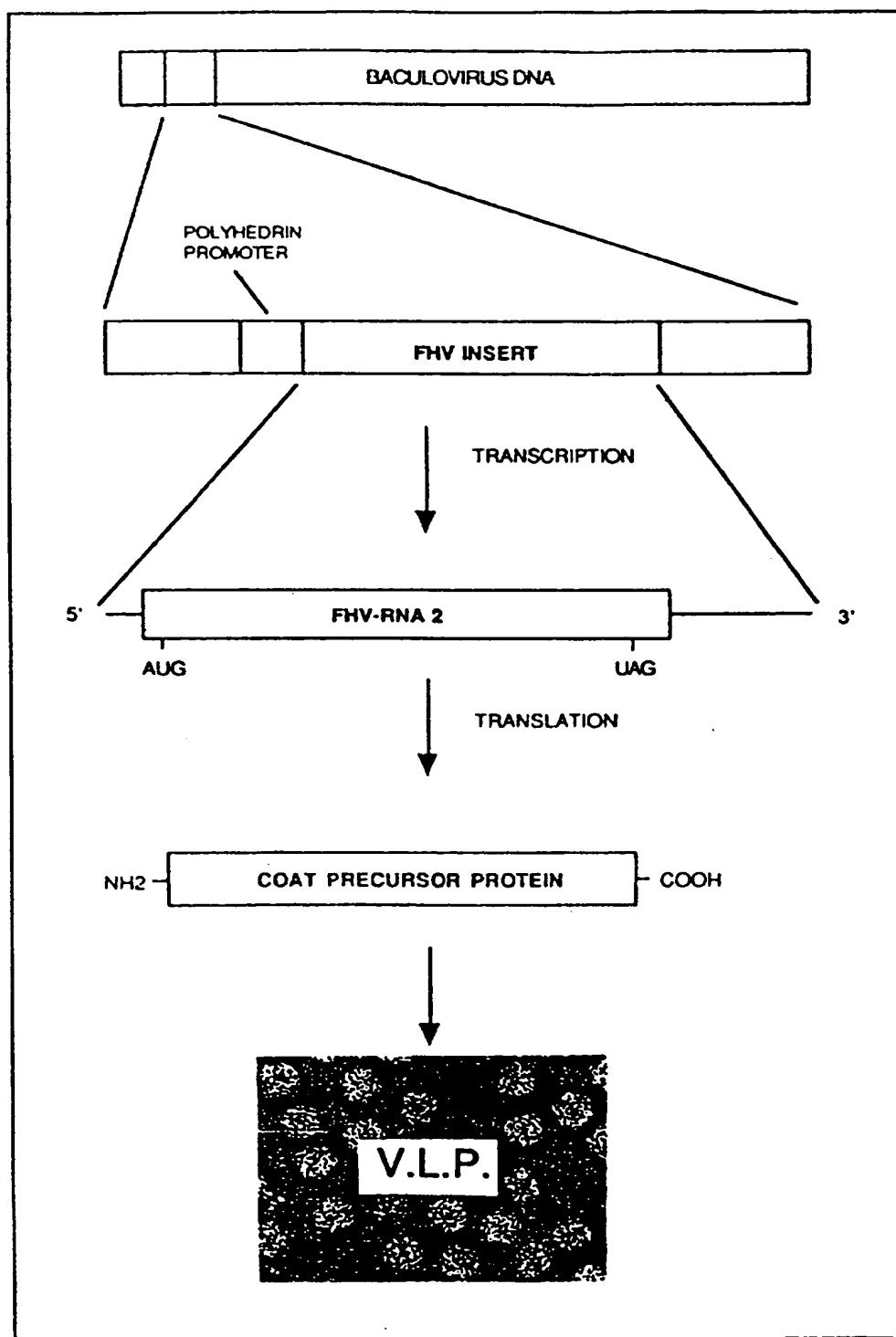
12/19

**FIG.7** Stereo diagram of the FHV protein showing the positions in which the HIV-1 specific sequence "IGPGRAF" is inserted.



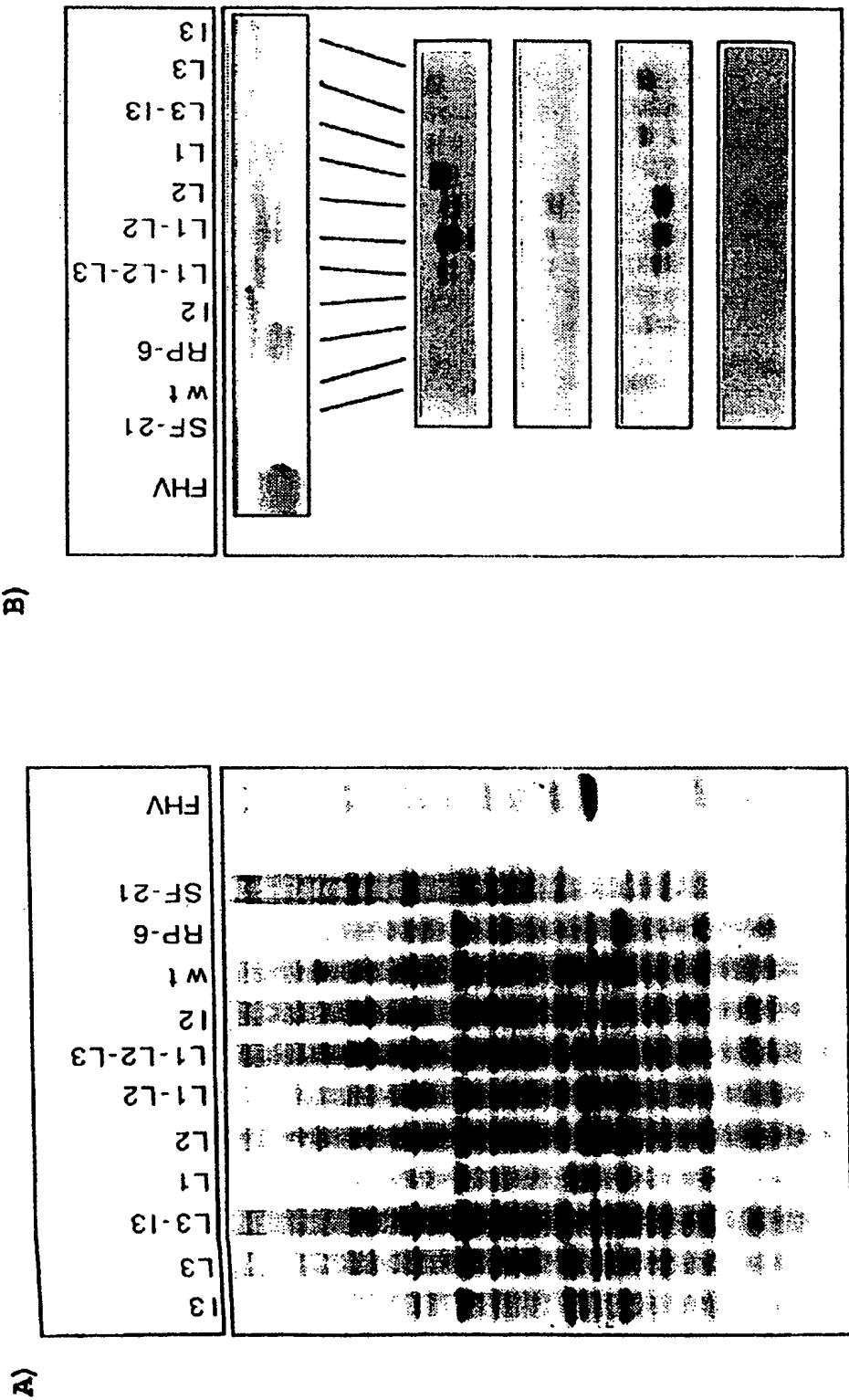
13/19

**FIG. 8** FHV Capsomer virus-like particles produced in Baculovirus.

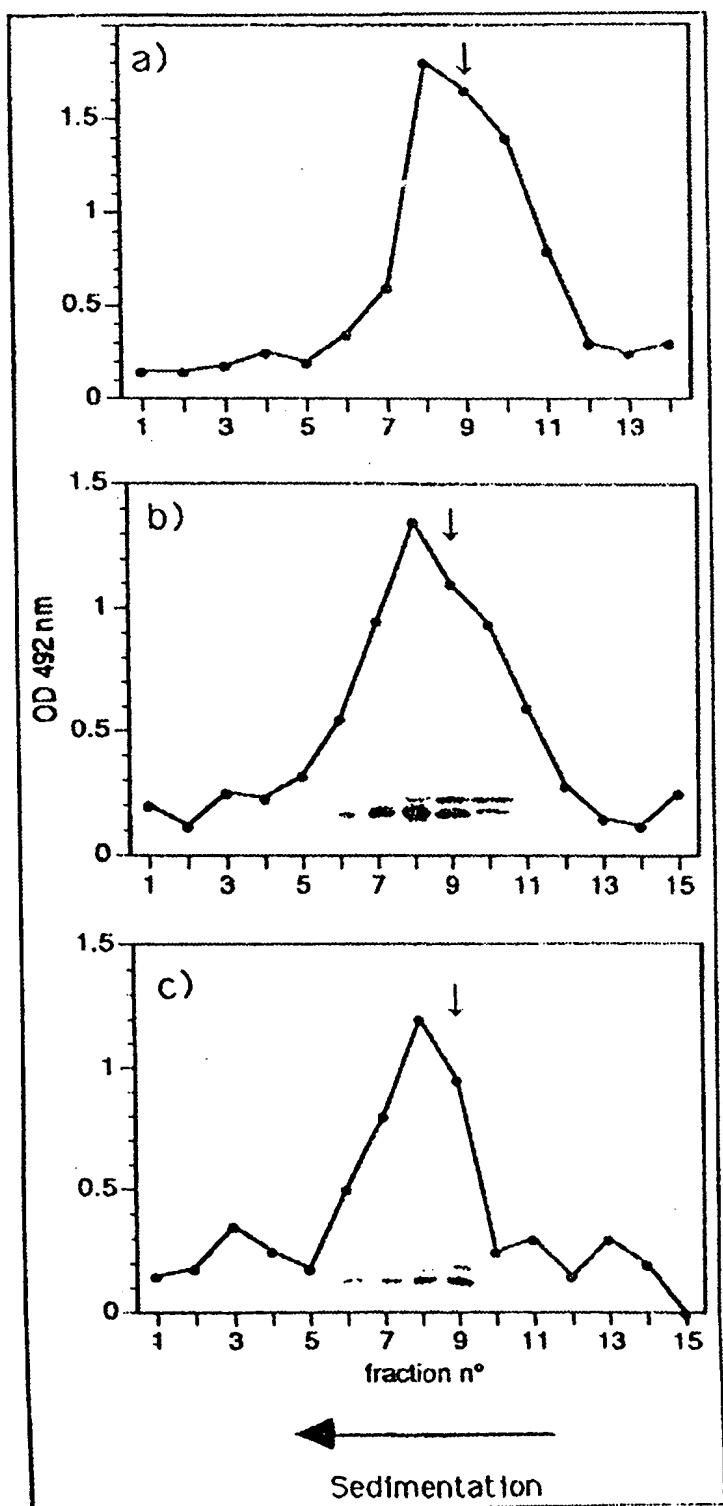


14/19

**FIG.9** Expression of the wild-type and the hybrid FHV capsomer. SF-21 infected cell lysates were analyzed in 10% SDS-PAGE. **A)** Coomassie stained. **B)** Western blot analyzed with: (1) antiserum against FHV. (2) human HIV-1 mAb. (3 and 4) HIV-1 positive sera and (5) HIV-1 negative serum.



**FIG.10** Sedimentation profile and antigen composition of VLPs produced by three different recombinant Baculovirus infected SF-21 cells.

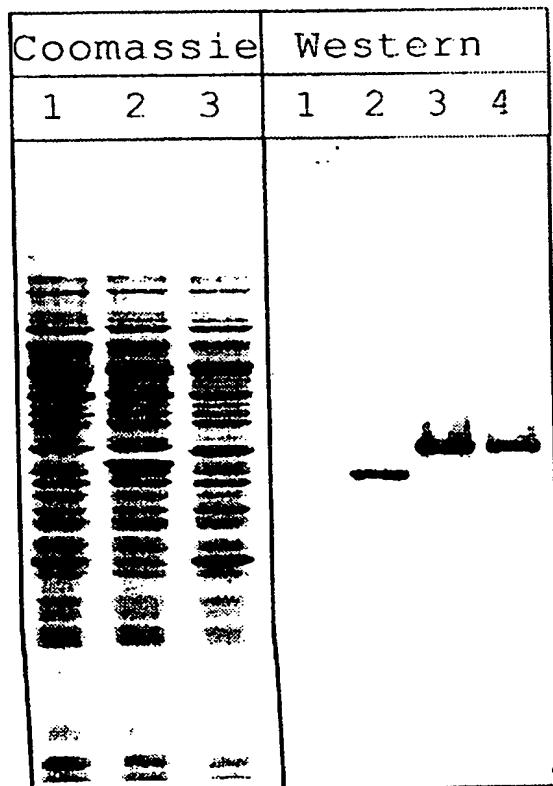


16/19

**Fig.11 Serum anti-V3 antibody titres measured against recombinant gp120**

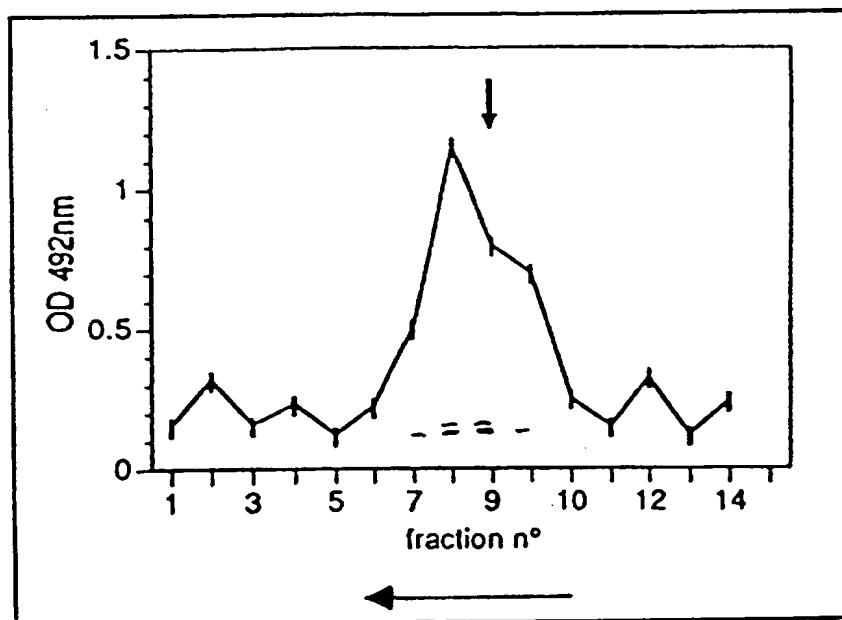
Ag. for imm. Ab titre	Ac NPV-V3 L3	Ac NPV-V3 I3	Ac NPV-V3 I3-L3
FHV	1:40000	1:20000	1:5000
	1:20000	1:60000	1:15000
	1:30000	1:10000	1:20000
gp 120	1:500	1:2000	1:250
	1:1500	1:5000	1:2000
	1:2000	1:1000	1:500

**FIG.12 Expression of hybrid FHV-HCVC protein through recombinant Baculovirus AcNPV-HCHc.**

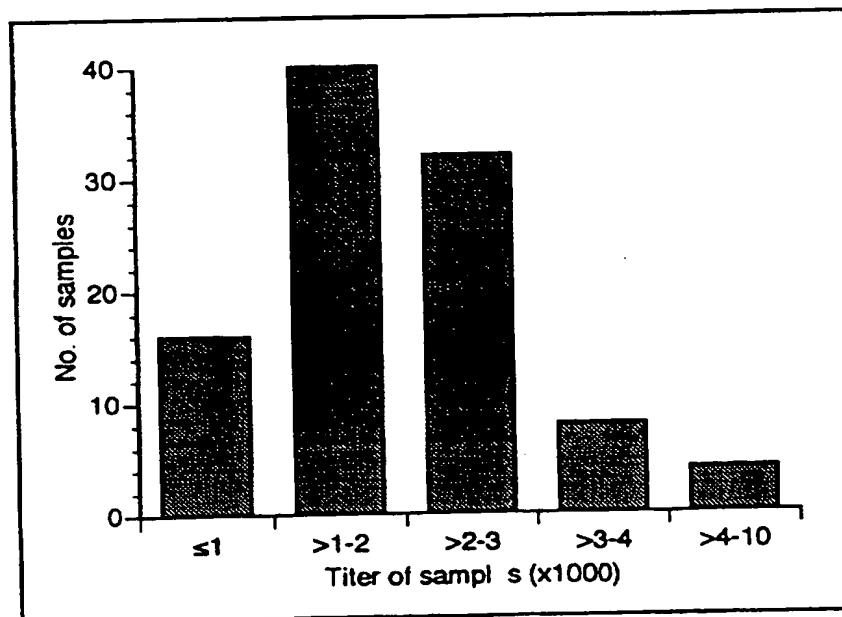


17/19

**FIG.13** Profile and FHV reactivity (ELISA test) after a sucrose gradient of VLPs formed by SF-21 infected cells with the recombinant Baculovirus AcNPV-HCV.

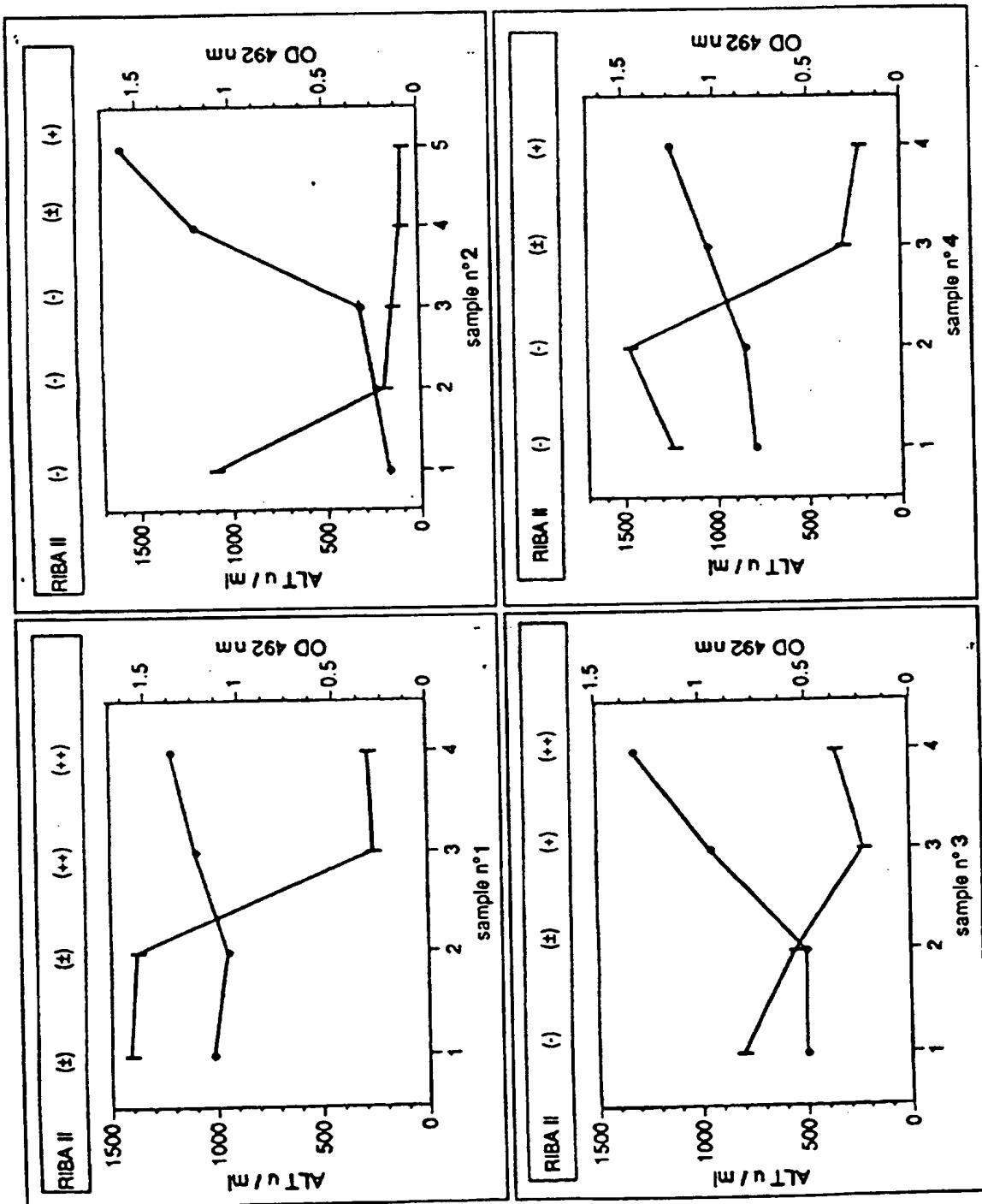


**FIG.14** Distribution of the ELISA titre obtained by a VLP based assay among 100 selected HCV core positive sera.



18/19

**FIG.15** Detection of HCV specific antibodies in a VLP based ELISA test and its correlation with ALT levels and previous RIBA II values.



19/19

**FIG. 16** Detection of HCV core-antibodies in human sera by dot-blots using recombinant (VLP-HCV) antigen or free peptides.

Patients							Antigen
1	2	3	4	5	6	NC	
							NS5-p
							NS4-p
							NS3-1-p
							NS3-2-p
							NS3-3-p
							HCC-1-p
							HCC-2-p
							pHCC-2

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 95/03114

A. CLASSIFICATION OF SUBJECT MATTER					
IPC 6	C12N7/00	C12N15/86	C12N15/62	C07K14/06	C07K14/16
	C07K14/18	A61K39/12	G01N33/53		

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. VIROL. (1993), 67(5), 2950-3 CODEN: JOVIAM; ISSN: 0022-538X, May 1993 FISHER, ANDREW J. ET AL 'Crystallization of viruslike particles assembled from flock house virus coat protein expressed in a baculovirus system' see the whole document ---	1,2
Y	J. VIROL. (1993), 67(5), 2756-63 CODEN: JOVIAM; ISSN: 0022-538X, May 1993 SCHNEEMANN, ANETTE ET AL 'Use of recombinant baculoviruses in synthesis of morphologically distinct viruslike particles of flock house virus, a nodavirus' see the whole document ---	1,2

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*&\* document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
4 January 1996	26. 01. 96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (- 31-70) 340-2040, Tx. 31 651 epo nl.  
Fax (- 31-70) 340-3016

Authorized officer

Hornig, H

**INTERNATIONAL SEARCH REPORT**

International Application No

PCT/EP 95/03114

**C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. VIROLOGY, vol. 66, no. 7, July 1992 AM.SOC.MICROBIOL., WASHINGTON, US, pages 4003-4012, B. ROVINSKI ET AL. 'Expression and characterization of genetically engineered human immunodeficiency virus-like particles containing modified envelope glycoproteins: Implications for development of a cross-protective AIDS vaccine' see the whole document ---	1,2
Y	J. GENERAL VIROLOGY, vol. 74, no. 7, July 1993 READING, BERKS, GB, pages 1261-1269, R. WAGNER ET AL. 'Induction of cytolytic T lymphocytes directed towards the V3 loop of the human immunodeficiency virus type 1 external glycoprotein gp120 by p55gag/V3 chimeric vaccinia viruses' see the whole document ---	1,2
A	NATURE, vol. 361, 14 January 1993 MACMILLAN JOURNALS LTD., LONDON, UK, pages 176-179, A.J. FISHER AND J.E. JOHNSON 'Ordered duplex RNA controls capsid architecture in an icosahedral animal virus' see the whole document ---	1-6
A	NUCLEIC ACIDS RES. (1989), 17(18), 7525-6 CODEN: NARHAD; ISSN: 0305-1048, 25 September 1989 DASGUPTA, RANJIT ET AL 'Nucleotide sequences of three Nodavirus RNA2's: the messengers for their coat protein precursors' see the whole document ---	1-6
P,X	FEBS LETT. (1994), 353(1), 1-4 CODEN: FEBLAL; ISSN: 0014-5793, October 1994 TISMINETZKY, SERGIO G. ET AL 'Immunoreactivity of chimeric proteins carrying the HIV-1 epitope IGPGRAPH. Correlation between predicted conformation and antigenicity' see the whole document ---	1-6
2		-/-

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 95/03114

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	VACCINE 13 (13). 1995. 1233-1239. ISSN: 0264-410X, September 1995 SCODELLER E A ET AL 'A new epitope presenting system displays a HIV-1 V3 loop sequence and induces neutralizing antibodies.' see the whole document -----	1-6